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PRINCIPAL INVESTIGATOR: Tian Xu

CONTRACTING ORGANIZATION: Yale University School of Medicine
New Haven, CT 06520

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Tian Xu, PhD

E-Mail: tian.xu@yale.edu

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University School of Medicine
Department of Genetics
295 Congress Avenue, BCMM RM 236
PO Box 208047
New Haven, CT 06520-8047

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14. ABSTRACT
The overall goal of the project is to identify driver mutations/genes that promote breast cancer progression. We have screened 70 genes, which are mutated in human breast cancer at low frequencies, by examining whether their piggyBac insertional mutants could promote the growth and metastasis of the MMTV-PyVT breast tumors in mice. We have discovered that 15 of them alter the onset of breast tumor formation while four exacerbate tumor metastasis. Among them, *Trim33* and *Ahrr* have been recently reported as tumor suppressors, demonstrating the effectiveness of our screen. We have further confirmed that the *Grik3* gene/mutation identified in the screen promotes breast cancer development by changing the onset and growth of the tumors. Our mechanistic studies have shown that *Grik3* regualtes the cell cycle, but not apoptosis, by inducing the expression of p53 and p21. In a complementary study, we have examined the role of tumor heterogeneity in contributing to TNBC invasion/metastasis. By co-culturing different breast cancer cells that are tagged with flurocent proteins, we have discovered that breast cells of different genotypes could promote invasion/metastasis behavior of other cells in a cell line-specific manner. Our study establishes a new paradigm for studying the contribution of tumor heterogeneity to breast cancer biology.

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INTRODUCTION:

Breast cancer is the most common malignant cancer among American women and the second leading cause of cancer death in women. These fatalities are due to the ability of later stage tumors to metastasize to distant sites in the body and form secondary tumors (1, 2). It is now well established that the development of cancer requires multiple genetic alterations (3). In recent years, the systematic analysis of genetic alterations in human cancers has revealed that individual tumors accumulate an average of ninety mutant genes (4). However, the significance of most acquired mutations in the development of cancer remains unknown. A key to deciphering the complexity of the cancer genome is to identify which mutations actually drive tumor development and progression to metastasis. These causative mutations and the pathways they affect are likely the effectors we must target with therapeutics to treat cancer. Thus, novel high throughput strategies are needed to identify and functionally characterize cancer genes. An effective approach to decipher the functions of human genes and test the role of these mutated genes in disease is to systematically mutate the orthologous genes in a mammalian model organism. This approach has not been feasible due to a lack of a large collection of mouse mutant strains. The completed mouse genome sequence has revealed that 99% of human genes have orthologs in the mouse (5). Many developmental processes, signal transduction and regulatory pathways, anatomical structures, and physiological and behavioral characteristics are also well conserved from mice to humans. To facilitate genome scale genetic analysis of human genes and to identify and verify human disease genes, we have initiated a large-scale mouse mutagenesis program at the Institute of Developmental Biology and Molecular Medicine (IDM) at Fudan University, Shanghai, China. This project has led to the establishment of more than 5,000 mutant mouse strains with genetically defined single gene mutations utilizing the *piggyBac* (*PB*) transposon system (6). Among these, we have identified seventy strains for which the orthologous human gene has been found to harbor mutations in breast cancer genome sequencing studies. We performed a screen to test the roles of these seventy genes in mammary tumorigenesis in the MMTV-PyVT transgenic mouse model. We have further characterized one of the identified genes, Grik3, and have found that it regualtes the cell cycle, but not apoptosis, by inducing the expression of p53 and p21.

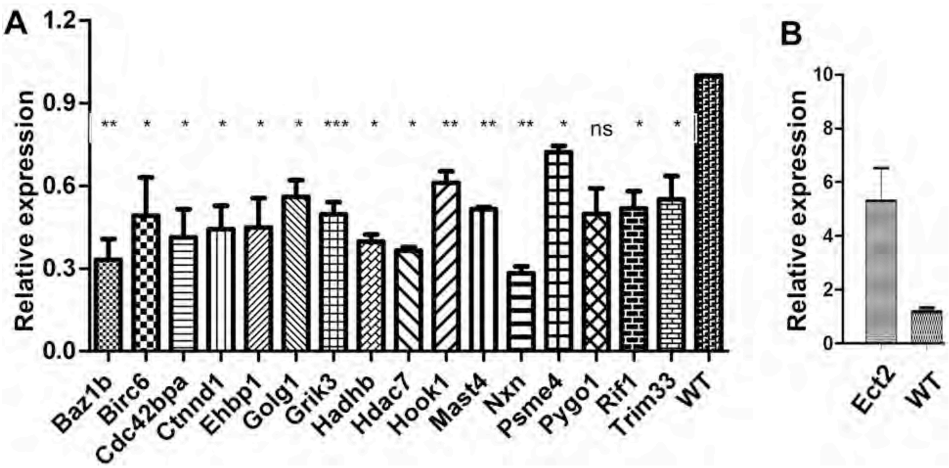
Because of the restriction for transfering fund to China, we could not do the many genetic crosses and screen outlined in our original proposal. Therefore, we have committed a part of our effort to develop a system to study breast cancer biology without the costly mouse genetics. We chose to focus on Triple-Negative Breast Cancer (TNBC) and have establsihed a new paradigm for studying the contribution of tumor herterogeneity to breast cancer biology.

BODY:

(1) To use our collection of *PB* mutant mouse strains to identify and characterize which mutations from human breast cancer samples are drivers of tumor initiation and progression.

Using the MMTV-PyVT mouse breast cancer model, we have performed a screen to test the roles of seventy breast cancer genes with *PB* insertional mutant mouse lines. Each *PB* mutant mouse line carries a mutant gene corresponding to a human ortholog mutated in human breast cancer. We identified and confirmed that that 19 genes altered breast tumor onset and four genes affected metastasis. Among the 19 genes, two of them, *Trim33* and *Ahrr*, have been recently reported as tumor suppressors, demonstrating the effectiveness of our screen (7, 8). We have examined the transcripts affected by the *PB* insertion in these strains and found that sixteen of them have significant reduction of expression while one has increased expression (Fig. 1). We then examined the dissected mammary pads and lungs from the MMTV-PyVT/ *PB* insertion mouse strains and

confirmed either the enhanced tumor growth or metastasis phenotypes.
Fig. 1. RT-qPCR results of Relative expression of 17 gene mutated PB insertion in 17 PB mutant mouse lines.



(2) The role of Grik3 in tumor development.

2A. Grik3 affects the onset of breast cancer development.

We have performed confirmatory experiments with the novel breast canncer gene, *Grik3,* which has been reported to be involved in metabolism. The *Grik3* gene encodes a subunit of glutamate receptor (also called kainate receptor). In the primary screen, eight *Grik3* mutant animals were exmained and the controls were not litte mates. We did confirmation experiment with 23 *Grik3* heterozygoous animals (Grick3$^{+/PB}$; MMT-VPyVT) and 25 wild-type litte mates as control (+/+; MMT-VPyVT). We have not only exmined the tumor onset (Fig. 2A and Fig. 3), but also dissected the tumors to confirm early onset of tumor development (Fig. 2B). We have also examined tumor burden and lung metastasis on these animals (Fig. 2C). We have found that heterozygous mutation *Grik3* in significantly promotes breast tumor developemnt (P<0.0006) and increases tumor burden, but does not affect metastasis.

Fig. 2. Heterozygout *Grik3* mutant affects breast tumor development. A. Breast tumor latency in Grick3$^{+/PB}$/MMT-VPyVT and control mice. B. Tumor burden. C. Lung metastasis.

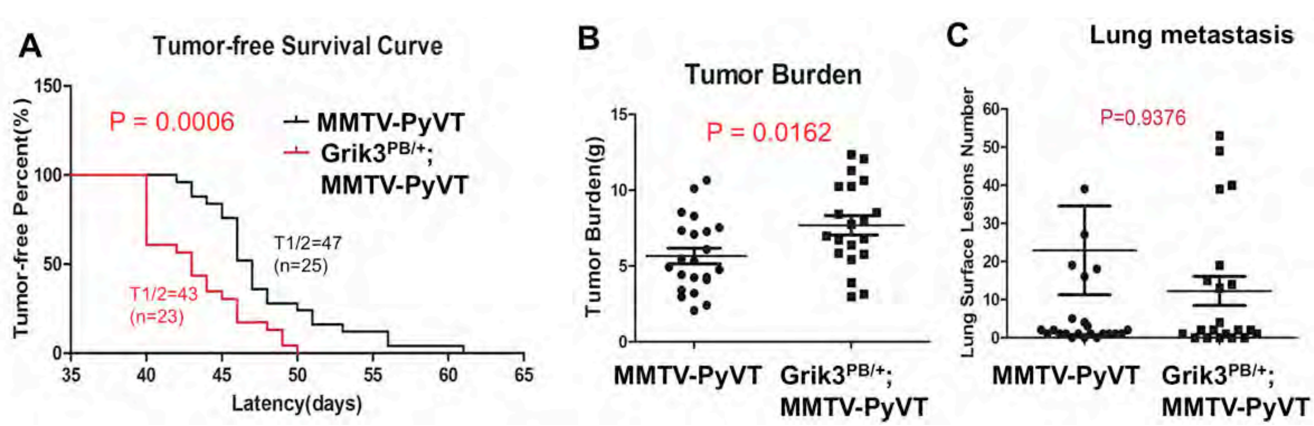
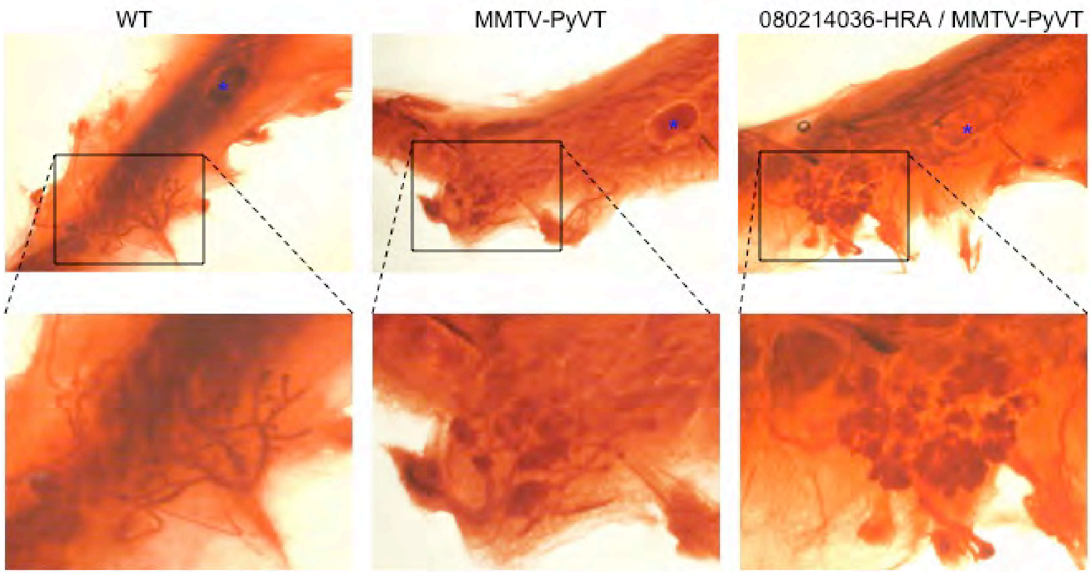


Fig. 3. Heterozygout *Grik3* mutant promotes breast tumor onset。 Microphotographes of dissected mammary fat pads from wt, MMT-VPyVT and Grick3$^{+/PB}$/MMT-VPyVT and control mice.



2B. Overexpression of Grik3 suppresses the growth and tumorigenesis of NIH3T3/v-Ras cells.

We have generated a viral expression system for *Grik3* overexpression*.* Using this system, we have examined that the effect of overexpression of *Grik3* on cell proliferation, cell cycle regulation, and apoptosis in the NIH3T3-v-Ras cells. We found that overexpression of *Grik3* suppresses cell proliferation (Fig. 4A), but not affect apoptosis (Fig. 5). Overexpression of *Grik3* alters the cell cycle. There are significantly more G1 cells while a significant reduction in S cells (Fig. 4B and 4C). G2/M cells are doubed (Fig. 4B and 4C).

We have also found that Overexpression of *Grik3* suppresses anchorage-independent growth of the NIH3T3/v-Ras in soft agar assay (Fig. 6A) and the tumorigenesis ability of the cells in nude mice (Fig. 6B).

Fig. 4. Grik3 inhibits proliferation of NIH3T3/v-Ras cells. Exogenous Grik3is expressed in NIH3T3/v-Ras cells. A. Growth curve. B and C. FACS analysis and statistical analysis of Cell cycle of NIH3T3/v-Ras/Grik3 and control cells.

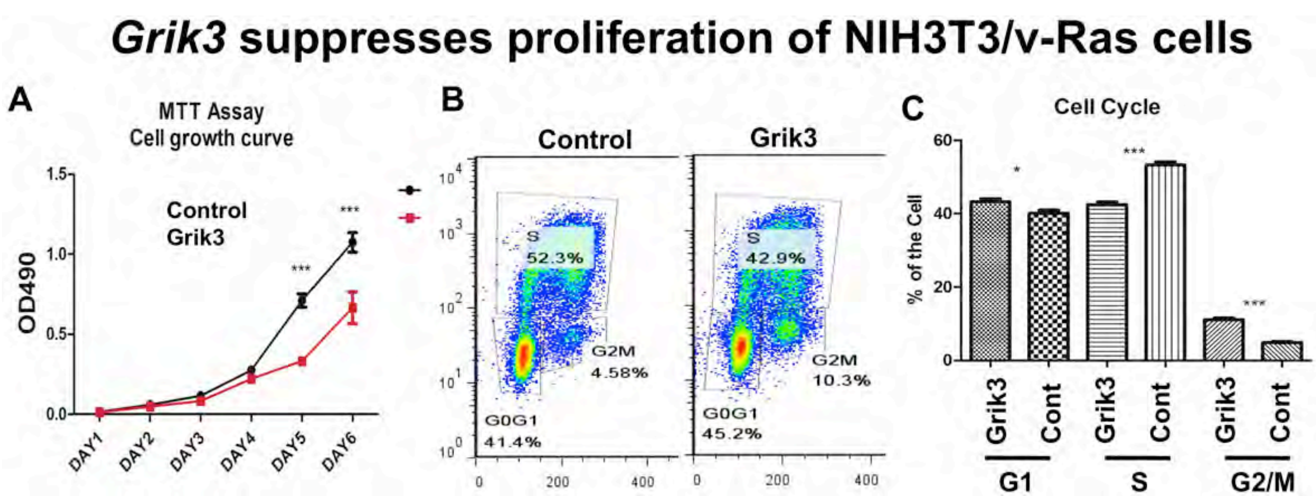


Fig. 5 *Grik3* inhibits proliferation of NIH3T3/v-Ras cells. Exogenous *Grik3is* expressed in NIH3T3/v-Ras cells. A. Growth curve. B and C. FACS analysis and statistical analysis of Cell cycle of NIH3T3/v-Ras/Grik3 and control cells.

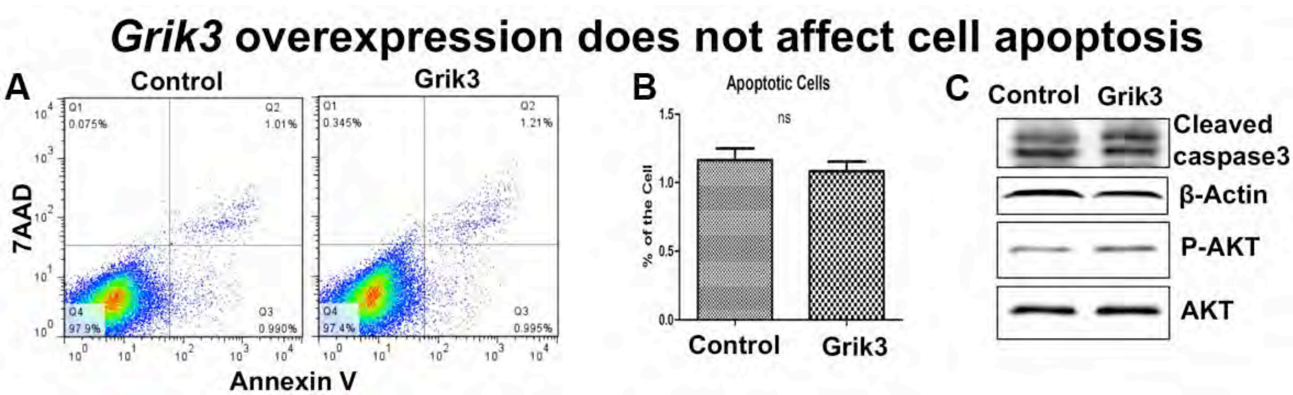
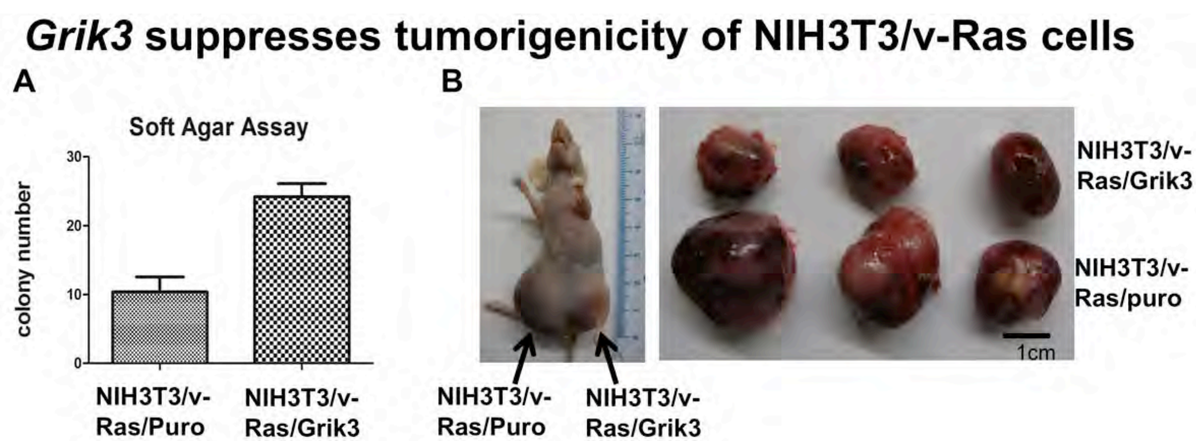


Fig 6. Grik3 suppresses tumorigenicity of NIH3T3/v-Ras cells in vitro and in vivo. A. colony number of NIH3T3/v-Ras/Grik3 and control cells in soft agar colony formation assay. B. tumor development of NIH3T3/v-Ras/Grik3 and control cells in nude mice.



(3) The molecular mechanism for Grik3's role in tumor development.

3A. Overexpression of Grik3 induces the expression of p53 and p21.

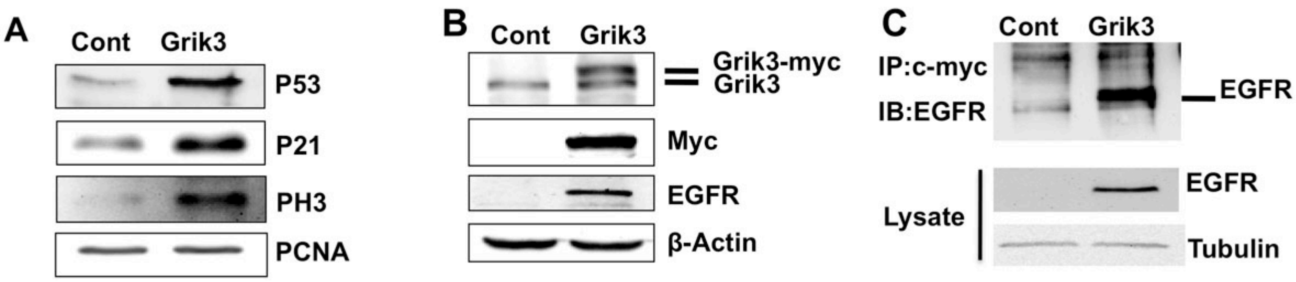
Consistent with cell proliferation and cell cycle phenotypes, we have found that overexpression of Grik3 induces the expression of the p53 gene and the p21 gene in NIH3T3/v-Ras cells and that the protein levels of p53 and p21 are also increased (Fig. 7A). These data indicate that Grik3 affects tumorigenesis by regulating the expression of p53 and p21.

3B. Grik3 complexes with EGFR.

Interestly, we have also found that overexpression of Grik3 induces the expression of the EGFR as well (Fig. 7B). Unexpectly, we found that Grik3 protein can be co-immunoprecipitated with

the EGFR protein (Fig. 7C). These data suggest that Grik3 regulates proliferation through multiple mecahnisms and it could be a novel regulator of EGFR signaling.

Fig. 7. Overexpression of Grik3 upregulates the expression of P53, p21 (A) and EGFR (B). C. co-immunoprecipitation of Grik3 with EGFR.



(4) Analyzing the Influence of Tumor Heterogeneity on TNBC Biology

TNBC accounts for 11-20% of all breast tumors and is defined by the lack of expression of the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor HER2 (7). Targeted therapies have increased survival rates in hormone receptor positive subtypes of breast cancer, but such effective agents remain elusive for TNBC. Some early stage TNBCs are highly sensitive to chemotherapy and achieve pathological complete response when treated with preoperative chemotherapy and they are often cured (8). Unfortunately, the majority has less sensitivity and many subsequenty develop aggressive metastatic disease (9). As a result, TNBC accounts for a disproportionate number of breast cancer deaths (7). No new therapeutic improvements occurred in the treatment of early stage TNBC since the introduction of paclitaxel 20 years ago. Thus, there is an urgent need to improve our understanding of TNBC cancer biology which could pave the way for more effective therapeutic intervention in TNBC.

Intratumor genetic heterogeneity has been detected in many different cancer types including TNBC and may be explained by the accumulation of different genetic changes in different cells during tumor growth (10, 11). There is increasing evidence that clonal diversity and clonal co-operation contributes to the clinical behavior and even to the formation of cancer. We were the first to demonstrate the proof of principle that inter-clonal interaction that can drive tumor formation and progression in *Drosophila* (12). Specifically, we found that cytokines released from one population of cells harboring tumor suppressor mutation can drive tumor development and progression of second population expressing oncogenic RAS. A similar inter-clonal relationship has been demonstrated in human glioblastoma that clarifies why these tumors commonly express truncating mutations and amplified EGFR in distinct clones (13). It was found that EGFR truncation induces the expression of the cytokines IL-6 and LIF, which drive the expansion of clones with amplified EGFR. Thus, paracrine signals between genetically distinct clones can drive tumor growth and progression.

In breast cancer, multiple coexisting clones have been distinguished by mutational composition and gene copy number variation (14-17) that provide the basis for possible clonal cooperation. Clinical experience indicate substantial clonal cooperation in breast cancer; the best example is that anti-estrogen therapy often produces tumor response or lead to growth arrest in cancers with as little as 10% of Estrogen Receptor (ER)-positive cancer (18). Tumor response and clinical benefit in this clinical scenario implies that there is a large bystander effect. Similarly, targeted therapy works for tumors with HER2 amplification despite heterogeneity for HER2 amplification within a cancer (19). These observations indicate that a minority of cells must interact with the rest of the tumor cells to maintain tumor survival or support tumor growth. Given the extensive tumor heterogeneity in TNBC, inter-clonal interaction could contribute to their growth and therapeutic resistance. This possibility has not been studied in the past.

4a. Tagging TNBC cell lines with multiple fluorecence proteins

We have examined about two dozens of TNBC cell lines for their behaviors in tissue culture and have chosen a panel of four TNBC cell lines including MDA-MB-453, HCC38, MDA-MB-231, and MDA-MB-436 for our pilot experiments. The TNBC cell line MDA-MB-453 has limited capacity for anchorage independent growth and cannot form tumors in immune-compromised mice. The TNBC cell line HCC38 can grow in immune-compromised mice while MDA-MB-231 and MDA-MB-436 cell lines can grow large metatstic tumors in immune-compromised mice. Using using matrigel invasion assay in triplicate utilizing FluoroBlok (BD Biosciences), we have foud that these four cell lines exhibit a nice range of invasion behavior: MDA-MB-453 (no invasion), MDA-MB-436 (low level of invasion), HCC38 (medium level of invasion) and MDA-MB-231 (high level of Invasion). This allows us to test whether co-culture of different cell lines could modify the invasion behavior.

These cell lines have been sequenced and transcriptionally profiled by our collaborator, Dr. Lajos Pusztai, Director of Yale Breast Cancer Program, and other investigators. We have functionally annotated these cell lines for growth sensitivity to siRNA knockdown, which will enable rapid functional annotation and mechanistic dissection of phenotypes in the future. We have stably labeled these cell lines with both the green fluorescent protein (GFP) and the the red fluorescent protein (RFP) markers, separately, through the piggyBac transposon expression constructs constitutively driving GFP or RFP.

4b. Inter-clonal interactions that promote invasion.

We have examined if inter-clonal interaction can enhance the invasive properties of these cell lines. Stably GFP or RFP-labeled cells were co-cultured with one of the four unlabeled cell lines and subjected to in a matrigel invasion assay in triplicate utilizing FluoroBlok (BD Biosciences). This assay is similar to a standard matrigel assay except a fluorescently blocking membrane is used to shield fluorescent signal from the top chamber until a fluorescently-labeled cell migrates through the matrix and membrane into the bottom chamber where fluorescence can be quantitated by a plate reader eighteen hours later. Again the GFP or RFP-labeled/unlabeled parental cell line pair was serve as baseline and increased invasion as measured by a significant increase in fluorescence signal is considered clonal cooperation.

The results are very exciting. We did observe clonal cooperation in a cell line-specific manner for both the receiver and promoter behaviors (Table 1; Fig. 8). For the behavior of promoting invasion, while HCC38 is a non-promoter of invasion for all, MDA-MB-453 is a promoter for all. MDA-MB-231 and MDA-MB-436 are selective promoters. MDA-MB-231 promotes invasion of MDA-MB-436, but not that of MDA-MB-453 and HCC38. MDA-MB-436 promotes invasion of HCC38 and MDA-MB-231, but not that of MDA-MB-453. For the behavior of receptive to being able to invade, while MDA-MB-453 is a non-receiver for all, both HCC38 and MDA-MB-231 are a receptive receiver for MDA-MB-453 or MDA-MB-436. MDA-MB-436 is a receptive receiver for MDA-MB-231 or MDA-MB-453, but not for HCC38. Both the promoter and receiver patterns are complicated and yet are highly reproducible and

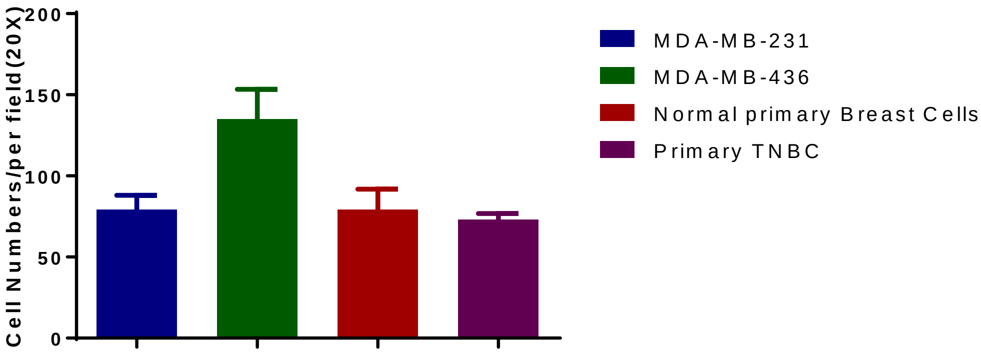
cell line-specific. First, these data show that breast cancer cells do able influrence other cells' behavior, strongly arguing that tumor heterogeneity plays crtical roles in tumor development and progression. The specific behavior patterns and the separation of receiving and promoting behaviors indicate specific underlying molecular mechanisms, most likely cytokines/ligands – receptors type of mechanism.

These data open a new paradigm for us to studying breast cancer biology. We are currently pursuing several lines of work. First, we are tagging more TNBC cell lines to examine their inter-clonal interaction on invasion. Second, we are using different tagged pair of cells in nude mice experiments to validate the results in vivo. Third, we are using conditioned media (media used in culture promoting cells) to see whether secreted factors are involved. Forth, we are doing multiple experiments to identify the molecules that are involved in these behaviors. We are doing informatic analysis to analyze gene expression in these cells to identify candidate genes and will then test these candidate genes in experiments by either providing the ligand or knockdown the receptor. We will fractionate the capable condition medium to biochemical identify the ligands. Our results on invasion have also open the door for exploring the cooperation on other beaviors including anchorage independent growth and migration.

Table 1. Invasion results from co-cultured experiments.

Tested Cells (Tagged)	Co-culture Cells	Invasion results
MDA-MB-453	MDA-MB-453	No Inv
MDA-MB-453	MDA-MB-231	No Inv
MDA-MB-453	HCC38	No Inv
MDA-MB-453	MDA-MB-436	No Inv
HCC38	HCC38	Med Inv
HCC38	MDA-MB-231	Med Inv
HCC38	MDA-MB-453	igh Inv, 3X
HCC38	MDA-MB-436	High Inv, 2.5X
MDA-MB-231	MDA-MB-231	High Inv
MDA-MB-231	HCC38	High Inv
MDA-MB-231	MDA-MB-453	Very High Inv, 2x
MDA-MB-231	MDA-MB-436	Very High, 2x
MDA-MB-436	MDA-MB-436	Low Inv
MDA-MB-436	HCC38	Low Inv
MDA-MB-436	MDA-MB-231	Med Inv, 5X
MDA-MB-436	MDA-MB-453	Med Inv, 2X

Fig. 8. MDA-MB-231 cells were promoted for invasion by co-cultured MDA-MB-436 cells, but do not by co-cultured normal primary breast cells or a random primary TNBC.



KEY RESEARCH ACCOMPLISHMENTS:

- **Screening *PB* mutant mouse strains we identified 15 genes that altered breast tumor onset and four genes that affected metastasis.**

- **Confirmed that the *Grik3* gene behaves as a tumor suppressor gene in tumorigenic assays.**

- **Discovered that Grik3 is a novel regulator of p53, p21 and EGFR.**

- **Developed a novel tissue culture-based system for studying the contribution of tumor heterogeneity to breast cancer invasion.**

REPORTABLE OUTCOMES:

Animal Models:
We have established *PB* insertional mutation lines for 19 genes that influence tumor onset in the MMTV-PyVT background.

Cell Lines:
We have established the GFP and RFP labeled MDA-MB-453, HCC38, MDA-MB-231, and MDA-MB-436 cells.

Manuscript:
We are in the process of preparing the manuscript describing the results of the piggyBac modifier screen for breast cancer in mouse model and the discovery of the molecular mecahnism underlying the role of the Grik3 gene in tumor development.

INVENTIONS, PATENTS, LICENSES:

None

CONCLUSION:

In summary, our *PB* insertional mutation strains have allowed us to rapidly functionally test breast caner mutations and identified a collection of novel breast cancer driver genes. Our approach has been validated by the independent verification of two of the identified genes from our screen, *Trim33* and *Ahrr*, by other groups (7, 8), and by our own characterization of the Grik3 gene. We believe that our innovative approach provides an effective *in vivo* assay to distinguish breast cancer driving mutations from those that are merely bystanders. Our characterization of the Grik3 gene has revealed that it is a novel regulator of p53, p21 and EGFR. Finally, our tissue culture-base study of the cooperation of TNBC cell lines in promoting invasion establishes a new paradigm for studying the contribution of tumor heterogeneity to breast cancer biology.

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APPENDICES:

None